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Respiratory and hematological responses of the bonnethead shark, *Sphyrna tiburo*, to acute changes in dissolved oxygen

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Abstract

Behavioral and physiological responses to acute changes in dissolved oxygen were examined in the bonnethead shark, *Sphyrna tiburo*. In two sets of respirometry experiments, sharks were randomly exposed to seawater in oxygen contents of 6.0, 5.0, 4.0 and 3.0 mg l^{-1}. During exposure, bonnetheads increased mouth gape from 0.8 cm at 6.0 mg l^{-1} to 2.2 cm at 3.0 mg l^{-1}, while ventilation volume increased from 0.61 to 5.28 l min^{-1} kg^{-1}. Standard oxygen consumption remained unchanged (163–181 mg O_2 kg^{-1} h^{-1}) throughout all treatments and was not significantly different. Utilization (%) declined from 52.3% at 6.0 mg l^{-1} to 21.3% when oxygen levels reached 3.0 mg l^{-1}. Changes in oxygen content of ambient water also caused no significant change in either blood oxygen content or hematocrit. Using cellulose acetate electrophoresis, a single hemoglobin profile was identified at seawater of 6.0 mg l^{-1} and hypoxic conditions. Results suggest bonnetheads are physiologically able to tolerate moderate levels of hypoxia.

Keywords: Shark; Hypoxia; Respiration; Ventilation volume; Utilization; Hemoglobin

1. Introduction

Aquatic environments exhibit large spatial and temporal variations in physical and chemical factors that can influence the physiology of fishes. Among the most common

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changes in chemical factors in marine environments is the typical decrease in availability of oxygen (hypoxia). In general, the simplest way for fishes to avoid acute hypoxia is to leave the area. However, in some instances, an escape behavior is not feasible or necessary, and fishes respond by employing a combination of behavioral and physiological strategies designed to maintain oxygen delivery to the tissues. These compensatory mechanisms are diverse and can include combinations of increasing ventilation volume, decreasing respiration and heart rate, increasing oxygen-carrying capacity by increasing hematocrit and increasing blood-oxygen affinity through differences in hemoglobin properties or through multiple variants of hemoglobin (review in Jensen et al., 1994).

Bonnetheads, *Sphyrna tiburo*, are obligate ram-ventilating sharks commonly found in shallow coastal waters throughout the Gulf of Mexico (Parsons, 1990). Bonnethead sharks are a benthic predator that have been observed on shallow sea grass flats (1–2 m) where dissolved oxygen levels have been recorded as low as 2.0 mg l^{-1} (Parsons, 1987; Carlson, unpublished information). In previous laboratory investigations, bonnethead sharks exhibited an increase in swimming speed and mouth gape in response to dissolved oxygen concentrations below 4.5 mg l^{-1} (Parsons and Carlson, 1998; Carlson and Parsons, 2001). Although these observed behavioral changes were assumed to increase ventilation volume and thus maintain oxygen supply to the tissues, no studies have demonstrated whether these behaviors are effective. The objectives of this study were to quantify and compare the respiratory and hematological responses of bonnethead sharks under normoxic and hypoxic conditions. We examined standard oxygen consumption, ventilation volume, gape, utilization, hematocrit, in vitro concentration of arterial oxygen and hemoglobin polymorphism.

2. Materials and methods

2.1. Collection, holding and experimental protocol

Bonnetheads (mean mass = 1.0 kg; ± 0.17 S.D.) were captured from the St. Andrew Bay System, FL during June–August 1997 using gillnets and transported to the National Marine Fisheries Service Laboratory, Panama City, FL. Sharks were held outdoors, in shaded, circular 3000-l tanks at 28.5 ± 0.5 S.E. °C and 30.1 ± 0.3 S.E. ppt with a flow-through seawater system for 1 month and fed every other day (ad libitum) on a diet of squid or fish. Prior to experimentation, sharks were not fed for 96 h to achieve a post-absorptive state (Parsons, 1990).

Sharks ($n = 8$) were anesthetized using 100 mg l^{-1} MS-222 (tricaine methanosulfate). A 20-ga hypodermic needle was inserted just posterior to the first dorsal fin. Following methods in Bushnell and Brill (1992), approximately 0.1 cc of lidophrine (20 mg lidocaine hydrochloride, 0.01 mg epinephrine) was injected into the spinal cord. This procedure was designed to paralyze all spinal motor nerves but leave all cranial nerves intact.

Sharks were placed upright in a 125-l polyethylene, open, flow-through respirometer and secured with velcro straps attached to the respirometer floor. Sharks were positioned in front of a 1.0-cm diameter pipe delivering seawater at approximately 17 l min^{-1}. Sharks were left undisturbed for at least 30 min to allow recovery from anesthesia. Seawater

pumped from a tank under normoxic conditions (herein defined as 6.0 ± 0.2 mg l^{-1}) was provided during recovery. Experiments were conducted at a seawater temperature of 28 °C and salinity of 30 ppt.

After recovery, each shark was exposed for 30–60 min to seawater at concentrations of 6.0, 5.0, 4.0 and 3.0 mg l^{-1} (± 0.2) delivered in random order from separate holding tanks. These seawater oxygen tensions were chosen based on previous experiments conducted by Parsons and Carlson (1998) and Carlson and Parsons (2001). Prior to each experiment, seawater within each holding tank was established and maintained at the appropriate dissolved oxygen level by bubbling nitrogen or oxygen through the water via diffuser stones. Dissolved oxygen levels within each tank were monitored with a YSI Model 51 B oxygen meter. The oxygen partial pressures of seawater within the respirometer were alternated by moving the pump from one tank to another and releasing a flow valve found in line. At each oxygen level, three replicate measurements of each variable were taken for each individual and pooled as a single mean for statistical analysis. Mouth gape (± 0.1 cm), taken from the tip of the snout to the lower jaw, was measured directly with a fixed ruler attached to the side of the respirometer.

Similar to the dye dilution technique developed by Jones et al. (1990) and Bushnell et al. (1990), a 50-cm long catheter constructed of PE-160 tubing attached to a 50-cc syringe was used to sample inspired ($P_{insp}O_2$) and expired ($P_{exp}O_2$) water. Inspired water was sampled directly anterior to the mouth after flushing the apparatus to ensure that no air bubbles were included. Expired water was sampled in a similar manner by inserting the end of the catheter about 3–4 mm into the third gill slit. Oxygen partial pressures were measured with a Microelectrodes polarographic oxygen electrode (Model MI-730) linked with a Linseis stripchart recorder connected in-line with the PE-160 tubing.

Utilization was calculated as:

$$U = [(P_{insp}O_2 - P_{exp}O_2)/P_{insp}O_2]*100$$

where U is utilization (%) and $P_{insp}O_2$ and $P_{exp}O_2$ are the O_2 partial pressures of the inspired and expired water, respectively.

Standard oxygen consumption (the amount of oxygen consumed at zero activity) was determined by flow-through respirometry as described in Fernandes and Rantin (1989). Oxygen tensions of ingoing (P_iO_2) and outgoing (P_eO_2) water in the respirometer were determined with a YSI Model 51B oxygen meter. Standard oxygen consumption was calculated as:

$$SVO_2 = V_r*\alpha*(P_iO_2 - P_eO_2)/w$$

where SVO_2 is standard oxygen consumption (mg O_2 kg^{-1} h^{-1}); V_r is the flow rate through the respirometer; α is the solubility coefficient for O_2 in seawater at the experimental temperature, pressure and salinity; P_iO_2 and P_eO_2 are the O_2 partial pressures of the ingoing and outgoing water, respectively; and w is the mass of the shark (kg).

Ventilation volume was calculated as:

$$V_g = SVO_2/(P_{insp}O_2 - P_{exp}O_2)*\alpha$$

Table 1
Mean behavioral, respiratory, and hematological variables (± S.E.) for bonnethead sharks measured at four dissolved oxygen levels (± 0.2 mg l^{-1})

Dissolved oxygen (mg l^{-1})	Gape (cm)	V_g (l min^{-1} kg^{-1})	U (%)	VO_2 (mg O_2 kg^{-1} h^{-1})	Hct (%)	ml O_2/100 ml blood
6.0	0.8 ± 0.04	0.61 ± 0.08	52.3 ± 8.9	173.4 ± 11.3	19.9 ± 1.0	0.602 ± 0.08
5.0	1.3 ± 0.06	1.14 ± 0.25	38.7 ± 2.7	163.1 ± 12.2	21.2 ± 0.6	0.550 ± 0.12
4.0	1.7 ± 0.13	2.22 ± 0.67	31.3 ± 3.2	181.1 ± 15.0	20.3 ± 0.5	0.584 ± 0.12
3.0	2.2 ± 0.12	5.28 ± 1.51	21.3 ± 4.1	174.6 ± 9.4	22.2 ± 1.2	n/a

where V_g is ventilation volume (l min^{-1} kg^{-1}); SVO_2 is standard oxygen consumption (ml O_2 kg^{-1} h^{-1}).

Comparisons between dissolved oxygen levels and SVO_2, V_g, gape and U were made with a single-factor analysis of variance (ANOVA). The assumptions of normality and homogeneity of variance were tested using normal probability plots of residuals and plots of residuals vs. predicted values (Neter et al., 1990). If the data did not meet the assumptions, log transformations were performed following recommendations in Zar (1984). Rejection of the null hypothesis (H_0: variables measured at normoxia are the same at all three lower treatments; $\alpha = 0.05$) was followed by post hoc multiple comparisons using the Bonferroni method applicable when not all pairwise comparisons are of interest (Neter et al., 1990).

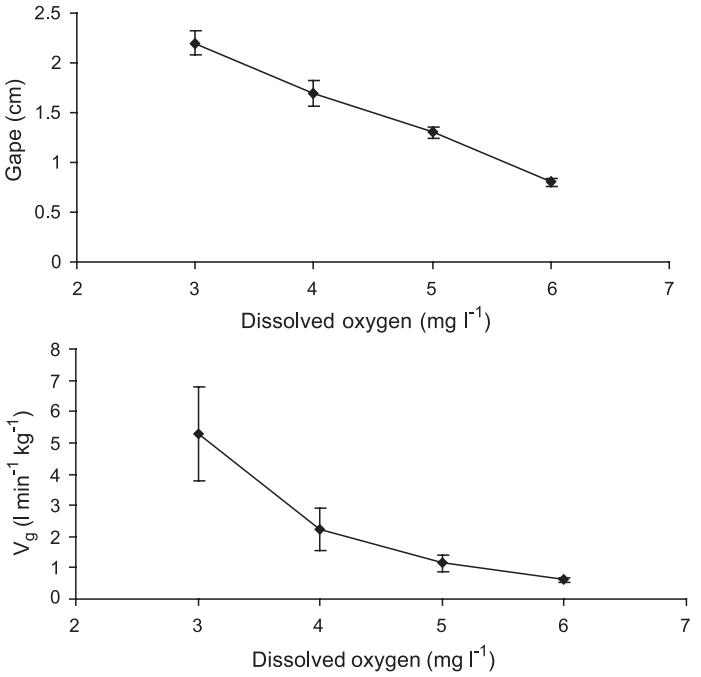

Fig. 1. Gape (cm) and ventilation volume (V_g; l min^{-1} kg^{-1}) as a function of dissolved oxygen level for bonnethead sharks held in stasis. Vertical bars represent ± 1 standard error.

In separate experiments, sharks ($n = 5$) were transferred from holding tanks immediately to 1750-l tanks containing either seawater at 28 °C and 30 ppt with dissolved oxygen concentrations of 6.0, 5.0, 4.0 or 3.0 mg l^{-1}. Sharks were allowed to swim freely within the tanks for 30–60 min.

After exposure, sharks were dipnetted from the tanks and blood (1.0 cc) was taken by caudal puncture with a 22 ga needle attached to heparinized syringes. Sampling was completed within approximately 30 s of first handling the shark. Blood oxygen content was determined immediately using an Oxycon blood oxygen content analyzer (Model OC100) at the experimental temperature, pressure and salinity.

A portion of the blood collected was transferred to three heparinized microhematocrit tubes per individual and centrifuged for 5 min at 14,000 G. Hematocrit was measured using Critocaps Micro-Hematocrit Capillary Tube Reader. At each oxygen level, three replicate hematocrit measurements were taken for each individual and pooled as a single mean for statistical analysis.

Hemoglobin polymorphism was determined in the laboratory from whole blood stored at 4 °C for 30–35 days. Stored blood samples were centrifuged for 5 min at 1000 G, and the supernatant was removed and discarded. Red blood cells were washed with 2.0 ml of phosphate buffered saline (PBS) and centrifuged. The supernatant was removed and discarded, then red blood cells were lysed using a hemolysate reagent (0.0005 M EDTA; 0.01 M potassium cyanide) and centrifuged for 5 min. Hemoglobin samples were

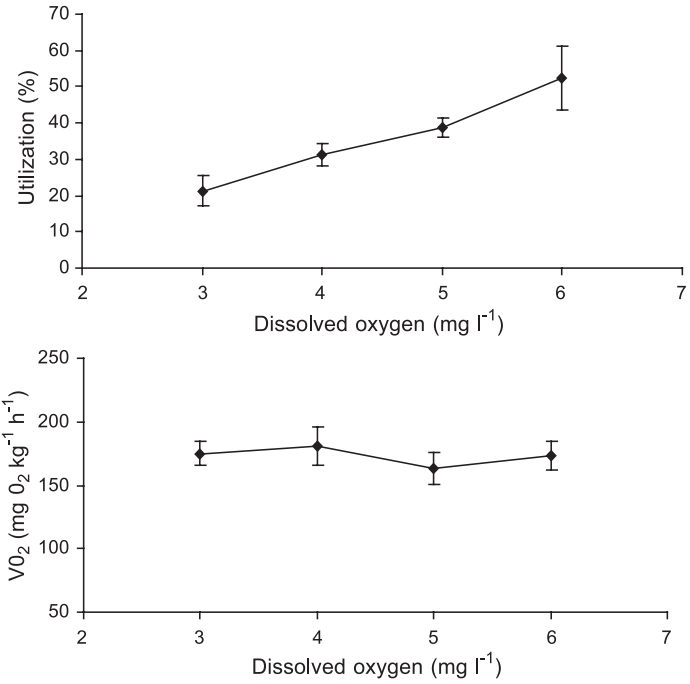

Fig. 2. Utilization (U; %) and standard oxygen consumption rate (mg O_2 kg^{-1} h^{-1}) as a function of dissolved oxygen level for bonnethead sharks held in stasis. Vertical bars represent ± 1 standard error.

separated using cellulose acetate electrophoresis plate in alkaline buffer (Tris–EDTA; boric acid; pH 8.2–8.6) procedure as described in Helena Hemoglobin Electrophoresis Procedure (Helena Laboratories, Beaumont, TX). Hemoglobin components were electrophoresed for 25 min at 350 V. Following electrophoresis, plates were stained with Ponceau S for 5 min and destained with 5% acetic acid for 2 min. Plates were washed in distilled water, stored at room temperature and later visually examined.

3. Results

Bonnetheads showed a significant increase in gape at all dissolved oxygen levels below normoxia ($F=34.86$, $df=3,32$, $p=0.0001$) (Table 1 and Fig. 1). Reductions in oxygen content caused an immediate increase in gape from 0.8 cm at normoxia to 1.3 at 5.0 mg l^{-1}, 1.7 at 4.0 mg l^{-1} and 2.2 at 3.0 mg l^{-1}. Similarly, ventilation volumes increased over all hypoxic values ($F=8.03$, $df=3,32$, $p=0.0004$) and were significantly different between normoxia and 4.0 mg l^{-1} and between normoxia and 3.0 mg^{-1}. V_g was not different between normoxia and 5.0 mg l^{-1} (Bonferroni post-hoc; $p>0.05$) (Fig. 1).

Standard oxygen consumption remained unchanged throughout all treatments and was not significantly different from normoxia ($F=0.782$, $df=3,30$, $p=0.51$). Oxygen con-

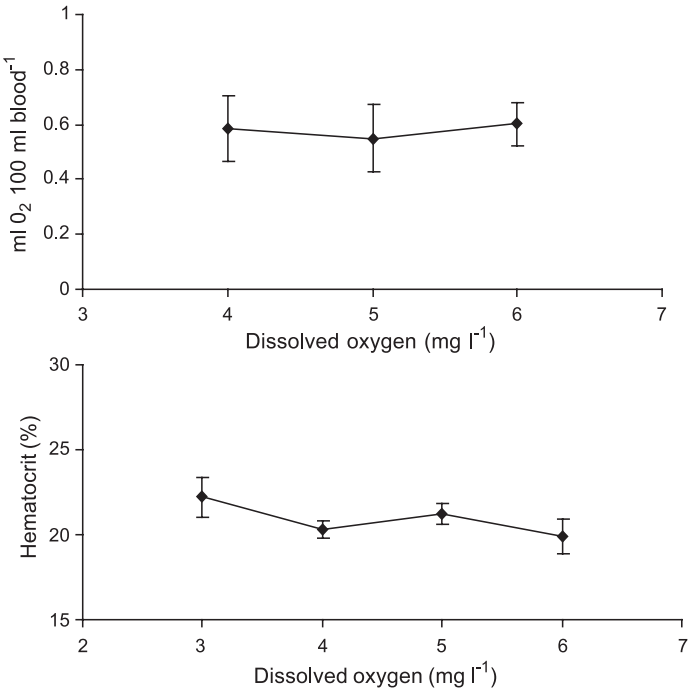

Fig. 3. Blood oxygen concentration (ml O_2 100 ml $blood^{-1}$) and hematocrit (%) as a function of dissolved oxygen level for swimming bonnethead sharks. Vertical bars represent ± 1 standard error.

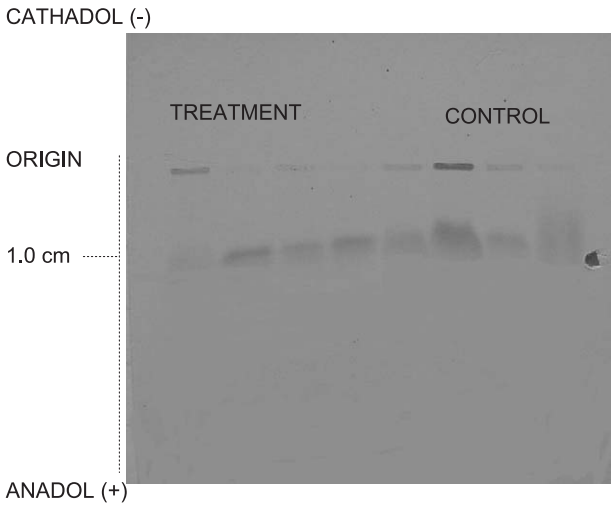

Fig. 4. Electrophoretic identification of hemoglobin bands for bonnethead sharks at normoxic and hypoxic conditions.

sumption varied between 163 mg O_2 kg^{-1} h^{-1} and 181 mg O_2 kg^{-1} h^{-1} (Table 1 and Fig. 2).

Exposure to acute hypoxia resulted in significant decreases in utilization ($F=8.94$, $df=3,32$, $p=0.0002$). Utilization (%) declined from 52.3% at 6.0 mg l^{-1} to 21.3% when oxygen levels reached 3.0 mg l^{-1} (Fig. 2). Significant differences were found only between normoxia and a hypoxic level of 3.0 mg l^{-1} (Bonferroni post-hoc; $p \leq 0.05$).

Due to logistic problems with the Oxycon blood oxygen content analyzer, blood oxygen content could be determined only for three levels of dissolved oxygen (Table 1). However, blood oxygen content and hematocrit in bonnethead sharks were similar throughout all dissolved oxygen levels (blood oxygen: $F=0.12$, $df=2,15$, $p=0.88$; Hct: $F=1.10$, $df=3,9$, $p=0.39$). Blood O_2 content varied between 0.55 and 0.60 ml O_2 100 ml $blood^{-1}$, while hematocrit varied between 19.9% and 22.2% (Fig. 3).

A single hemoglobin profile was identified under normoxic and under hypoxic conditions. The hemoglobin profile was anadol and traveled about 1.0 cm from origin (Fig. 4).

4. Discussion

Physiological studies on the cardiorespiratory system of obligate ram-ventilating fishes such as tunas or sharks should be conducted on fish swimming (Bushnell et al., 1990). However, the difficulties involved in obtaining simultaneous measures and with fish towing multiple cannulae make these types of studies complicated or impractical. We used the spinal block method (Bushnell et al., 1990), which selectively inhibits swimming muscle activity and which we believe yields representative results. McKim and Goeden

(1982) and McKim et al. (1987) employed a more severe method (severing the vertebrae near the dorsal fin) and reported no significant differences in VO_2, V_g, U or heart rate between severed and unsevered brook trout, *Salvelinus fontinalis*, and rainbow trout, *Salmo gairdneri*. Moreover, the measurement of standard oxygen consumption rate for spinal blocked bonnethead sharks obtained in this study was similar to that obtained by extrapolating to zero swimming speed (155.9 mg O_2 kg^{-1} h^{-1}) from a oxygen consumption rate-swimming speed regression reported in Carlson (1998).

Bonnethead sharks were sensitive to reduced dissolved oxygen. The response was rapid and lasted for as long as hypoxic conditions remained. An increase in gape was the most immediate adjustment to hypoxia, suggesting that receptors for low oxygen levels are found in the gills, rather than in the nervous system (Bamford, 1974). Milsom and Brill (1986), using in vitro perfusion of the first gill arch of yellowfin tuna, *Thunnus albacares*, also suggested the presence of an internal oxygen receptor in the gills.

Ventilation volume of bonnethead sharks at normoxia was higher than for buccal-ventilating elasmobranchs but did not approach that of ram-ventilating tunas. Piiper and Schumann (1967) measured a ventilation volume of 0.43 l min^{-1} kg^{-1} for lesser spotted dogfish, *Scyliorhinus stellaris*. Hanson and Johansen (1970) and Cameron et al. (1971) determined a ventilation volume of 0.10–0.29 and 0.17 l min^{-1} kg^{-1} for spiny dogfish, *Squalus acanthias*, respectively. Ventilation volumes for bonnethead sharks were 0.61 l min^{-1} kg^{-1} under normoxia in our study. Among teleosts under normoxic conditions, Kiceniuk and Jones (1977) measured a ventilation volume of 0.21 l min^{-1} kg^{-1} for rainbow trout, *Salmo gairdneri*, while ventilation volume measured by Bushnell and Brill (1992) for obligate ram-ventilating yellowfin and skipjack tunas, *Katsuwonus pelamis*, were 3.9 and 6.7 l min^{-1} kg^{-1}, respectively. All species had a similar utilization ($\sim$ 33–50%). Although bonnethead sharks and tunas are obligate ram-ventilators, the dramatic increase in ventilation volume for tunas is likely due to a higher metabolic rate (three to five times higher than teleosts) and the associated larger gill surface areas (Bushnell and Jones, 1994).

Increases in ventilation volume during hypoxia were inversely related to percent utilization. This relationship has been found in studies of buccal-ventilating teleosts such rainbow trout (Holeton and Randall, 1967; Hughes and Saunders, 1970), striped mullet, *Mugil cephalus* (Cech and Wohlschlag, 1973), tilapia, *Oreochromis niloticus* (Fernandes and Rantin, 1989), and erythrinids, *Hoplias malabaricus* and *H. lacerdae* (Rantin et al., 1992), as well as with skipjack and yellowfin tunas (Bushnell and Brill, 1992). Decreases in utilization under hypoxic conditions may be associated with an increased venous oxygen tension, increased dead space between gill filaments or secondary lamellae as the ventilation volume is shunted between filaments, or a reduced water contact with the respiratory surface (Randall, 1970; Jones and Randall, 1978).

Fishes commonly exposed to long periods of hypoxia demonstrate the most effective compensation for reductions in oxygen. Bullhead, *Ictalurus nebulosus*, sucker, *Catostomus carpio* (Saunders, 1962), carp, *Cyprinus carpio* (Lomholt and Johansen, 1979), and flounder, *Platichthys flesus* (Steffensen et al., 1982) increase ventilation volume while maintaining the same percent utilization found under normoxic conditions. The ability of these fish to maintain extraction efficiencies where others teleosts cannot may be related to species-specific oxygen binding properties of hemoglobin. Weber and DeWilde (1976)

determined that the oxygen affinity of hemoglobin for the hypoxia-tolerant flounder, *Platichthys flesus*, was higher than the less-tolerant plaice, *Pleuronectes platessa*.

This study found bonnethead sharks with hematocrit levels similar to that of other ectothermic sharks. Bushnell et al. (1982) reported hematocrit levels of 14.9% for lemon sharks, *Negaprion brevirostris*, at rest. In a comparative study, hematocrit levels were between 14.9% and 19.8% for sandbar, *Carcharhinus plumbeus*, dusky, *C. obscurus*, tiger, *Galeocerdo cuvier*, blue, *Prionace glauca*, and scalloped hammerhead, *Sphyrna lewini*, sharks while those for endothermic lamnid shortfin mako, *Isurus oxyrinchus*, and white, *Carcharodon carcharias*, sharks were 40.8% and 36.0%, respectively (Emery, 1986). Higher hematocrit levels for endothermic sharks such as these are not surprising given lamnid sharks possess several characteristics and adaptations indicating they have high aerobic and anaerobic capacities, and higher metabolic rates than ectothermic sharks (Bernal et al., 2001).

Increase in blood-oxygen affinity via increased hematocrit is a common adaptation observed in teleosts exposed to hypoxic conditions. Eel, *Anguilla anguilla*, exposed to water at oxygen levels of 40–50 mm Hg for 2 weeks increased blood-oxygen carrying capacity by increasing hematocrit and hemoglobin concentration (Wood and Johansen, 1973). Carp exposed to diurnal changes in hypoxic conditions also responded by increasing hematocrit and hemoglobin concentration (Lykkeboe and Weber, 1978). However, bonnetheads did not increase hematocrit in response to acute hypoxic conditions. Although long-term exposure to hypoxia may cause an increase in hematocrit, no studies have yet demonstrated an increase in hematocrit for elasmobranchs under hypoxic conditions (Short et al., 1979; Perry and Gilmour, 1996; Routley et al., 2002).

Hemoglobin polymorphism is common in fishes and has been suggested to be a response to living in environments with variable oxygen availability (review in Riggs, 1971; Powers, 1980). Cathadol components (pH insensitive; little or no Bohr shift), complementary to anadol components among their hemoglobin variants, have been postulated to aid fishes in oxygen uptake, as well as to provide an emergency back-up to allow continued swimming during transient periods of acidosis (Powers, 1972). In addition, fishes with multiple hemoglobins may have a physiological advantage in blood-gas transport (Giles and Randall, 1980). Qualitative analysis of bonnethead hemoglobin exhibited only one anadol hemoglobin variant. Hypoxia has been shown to elicit a change in hemoglobin component pattern in some fishes (Weber and Jensen, 1988; Marinsky et al., 1990) but not in others (Jensen and Weber, 1982). Final resolution of this issue for bonnetheads requires further investigation.

Marine teleosts tolerant of hypoxia generally have blood with a higher oxygen affinity (lower P_{50}) and a blood oxygen–disassociation curve that is shifted to the left. Although P_{50} values and blood oxygen–disassociation curves were not measured in this study, preliminary evidence suggests that bonnetheads are more tolerant of low ambient oxygen conditions than other shark species. Haggard (2000) determined an average P_{50} for two bonnetheads of 14.2 mm Hg at 28 °C, while comparable sized Atlantic sharpnose sharks, *Rhizoprionodon terraenovae*, have a P_{50} of 23.3 mm Hg at 27 °C. Although other information on shark blood characteristics (e.g. Bohr factor, cooperativity, oxygen capacity) is rare, sharks like the bonnethead which are specialized for continuous activity may have additional blood-oxygen binding properties, such as a large Bohr factor similar

to bat rays, *Myliobatis californica* (Hopkins and Cech, 1995). Future studies should investigate this possibility.

Increases in swimming speed and gape in response to hypoxia observed in bonnethead sharks have been proposed as mechanisms for increasing ventilation volume to the gills and consequently maintaining oxygen delivery to the tissues (Parsons and Carlson, 1998; Carlson and Parsons, 2001). Using spinally blocked sharks allowed for the measurement of some physiological variables (e.g. V_g and U) but this method precluded measurement on swimming sharks, which was proposed to work in conjunction with gape. However, partial support for sustaining oxygen supply to the tissues is supported based on the results from free-swimming bonnethead sharks in our study. Bonnetheads had a similar blood oxygen content at two levels of reduced dissolved oxygen but the full means by which sharks are accomplishing this need to be explored. In the short-term, sharks may also be altering heart rate, increasing stroke volume or relying on venous oxygen reserve. In addition, longer-term exposure to reduced oxygen may result in an increase in hemoglobin affinity, increases in hematocrit or hemoglobin polymorphism, which could increase oxygen uptake.

As discussed in Carlson and Parsons (2001), increasing swimming speed without an accompanying increase in gape would be ineffective at maintaining oxygen delivery. However, increasing swimming speed as a mechanism for regulating respiration appears to be metabolically costly since oxygen consumption rate increases with speed as a power function (Parsons, 1987; Carlson, 1998). Ventilation volume (i.e. oxygen delivery) would be predicted to increase with speed but oxygen demand would also increase, thus a point is reached where oxygen demand exceeds delivery. Oxygen delivery is likely met under moderate hypoxia where sharks swim at speeds between 30 and 35 cm s^{-1} (Carlson and Parsons, 2001), but a cost/benefit model incorporating demand with increases in gape, swimming speed, VO_2, V_g and decreases in U over a range of seawater dissolved oxygen concentrations is necessary to fully address this question.

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